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Effects of Exogenous Estrogenic Agents on Pubertal Growth and Reproductive System Maturation in Female Rhesus Monkeys

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Concern has been raised that environmental contaminants with estrogenic properties can alter normal sexual maturation. Monkeys, like humans, undergo a long and complex period of development during adolescence, which makes them important models for understanding exogenous estrogen effects during this period. This study examined the consequences of treatment with estrogenic agents (methoxychlor, MXC, 25 and 50 mg/kg/day; diethylstilbestrol, DES, 0.5 mg/kg/day) given in the peripubertal period (6 months before and after the expected age at menarche) to female rhesus monkeys. These treatments increased estrogen activity of serum as determined with an *in vitro* estrogen receptor alpha (ERa) transcription assay. DES completely suppressed adolescent growth (weight and height) and menses in a reversible manner; smaller effects of MXC on the timing of growth and menarche were also detected. Both DES and MXC led to premature emergence of a secondary sex characteristic, reddening and swelling of skin, but retarded growth of the nipple. As evaluated by ultrasound after an 8-month recovery period, uterine size was not affected by exogenous estrogen, but there was some indication of increased incidence of ovarian cysts/masses in MXC- and DES-treated groups. Ovarian cyclicity, as reflected in urinary hormone metabolites, demonstrated shorter follicular stages in the MXC-treated monkeys. In conclusion, the data indicate that DES had a striking effect on adolescent maturation and that the estrogenic pesticide MXC also altered development during this period. The pattern of effects across agents and doses may be based on specifics of estrogenic action, such as relative ERα and ERβ binding and activation. Long-term consequences of this disruption of pubertal development are being studied in this cohort of monkeys as adults.

Key Words: **endocrine disruption; environmental estrogen; nonhuman primates; puberty; adolescence; ovary; growth spurt.**

Concern has been raised about the adverse influence of environmental agents with estrogenic actions on reproduction. In addition to influences on adults, abnormal development of the reproductive organs and other organ systems that mature under the influence of gonadal hormones have come under discussion. Pubertal maturation is one of the areas of concern. In humans, environmental toxicants have been implicated in accelerated and delayed pubertal maturation of both males and females (Blanck *et al*., 2000; Colon *et al*., 2000; Den Hond *et al*., 2002).

Use of therapeutic estrogens at puberty is also a concern. Estradiol has long been used clinically to limit height growth of pubertal girls when height was seen as a psychosocial disadvantage (Barnard *et al*., 2002; Conte and Grumbach, 1978; de Waal *et al*., 1995). Data on long-term side effects are limited (Barnard *et al*., 2002; de Waal *et al*., 1995; Trygstad, 1986). Diethylstilbestrol (DES) has also been used for this purpose. A follow-up study is currently underway—the Tall Girls Study—of Australian women treated with DES between ages 8 and 16 (Venn, 2002). DES has also been used during puberty for the treatment of the genetic hypogonadal disorder Turner′s syndrome (Padmanabhan *et al*., 1988).

Monkeys, like humans, have a long and complex period of maturation during adolescence, which makes them important models for understanding exogenous estrogen effects during this period. Like humans, female nonhuman primates undergo a growth spurt and development of secondary sex characteristics (sex skin swelling and nipple growth), followed by menarche, intense bone mineralization and height growth, epiphyseal closure, and finally, the onset of stable ovulatory menstrual cycles (Tanner *et al*., 1990; Terasawa, 1995; Terasawa *et al*., 1983; Wilson, 1989, 1997). In female rhesus monkeys, this progression takes place between 24 and 42 months of age, comparable to 10–18 years of age in girls.

Research indicates that the onset of puberty is regulated differently in primates than in rodents (Mann and Plant, 2002; Terasawa and Fernandez, 2001). In female rhesus monkeys, hypothalamic-pituitary-gonadal (HPG) systems are functional in infancy but are then suppressed until the onset of puberty (Pohl *et al*., 1995; Terasawa, 1985). Prior to menarche, pulsatile gonadotropin releasing hormone (GnRH) release is initiated. After menarche, GnRH production increments gradually until the luteinizing hormone (LH) surge of ovulation occurs. Much of this maturational process is independent of endogenous estrogen and can be demonstrated in ovariectomized

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animals. In contrast, puberty in rats is marked by a change in hypothalamic sensitivity to estrogen negative feedback and does not include a juvenile inhibition stage. Estrogenic toxicants could be anticipated to act differently on these two types of systems.

In this study, exogenous estrogen effects on growth and sexual development at puberty were studied in a cohort of young female monkeys as they approached menarche. Effects on maturation of other systems (skeletal, immune, CNS) were also studied and will be reported separately. Methoxychlor (MXC) and DES were chosen as exogenous estrogens because of the large data base concerning their estrogenic actions in laboratory animals and, in the case of DES, in humans. In addition MXC, via its estrogenic metabolite hydroxyphenyl trichloroethane (HPTE), and DES have been well characterized for their estrogenic activity at the cellular and molecular level and are known to differ from 17β estradiol (E2) in specific ways.

MATERIALS AND METHODS

Subjects. Prepubertal female rhesus monkeys 20 ± 1 months of age were obtained from the breeding colony at the California National Primate Research Center (CNPRC). Prior to the experiment, monkeys lived in outdoor half-acre cages with 80–100 animals/cage. They were screened for medical and caging history to provide a uniform background. The original cohort contained 36 monkeys; four monkeys which showed the poorest adaptation were eliminated from the study prior to initiation of dosing.

Animal care and housing. After screening, monkeys were moved to indoor caging rooms and adapted for 4 months prior to initiation of dosing. They were housed in indoor colony rooms (48 animals per room) with individual light (lights on 0600 h to 1800 h) and temperature (68–78°F) regulation. A double cage (120 × 65 × 79 cm) with a partition allowed separation of pairs for food intake determinations and behavioral testing while permitting socialization at other times. The Enrichment Coordinator at the CNPRC determined compatible pairs based on prior cage location and individual temperament.

Purina monkey diet (Lab Diet #5047, PMI Nutrition International, Richmond, IN) was provided twice daily at 0700 and 1500 h. An algorithm was used to increase the amount of diet (biscuits/day) provided based on food consumption during this period of rapid growth. Manufacturer′s data indicated that the diet contained 220 ± 26 ppm genistein and 217 ± 21 ppm daidzen, measured as the aglycone form of the isoflavone. Monkeys also received supplementary foods as enrichment. These were carefully screened for plant estrogens and included apples, bananas, pears, kiwi, dried apricots, carrots, zucchini, cucumber, cherry tomatoes, and a granola mix. An automatic system with a spigot in each cage provided drinking water. Function of the spigots was checked daily. Monkeys were observed each morning for health signs and referred to the veterinarians for treatment, if needed. An individual medical record was maintained for each animal.

Experimental design. The cohort was divided into four groups of eight animals balanced for age, size, and caging history. Dosing was initiated at 24 ± 1 months of age and continued for 12 months. This period spanned 6 months before and after the anticipated average age at menarche at CNPRC (30 months). Subsequently, an 8-month recovery period was conducted. A series of evaluations of growth, reproductive tract maturation, skeletal growth and maturity, immunohematology, and behavioral function were conducted during the dosing and recovery periods.

Exogenous estrogen treatments. Monkeys (n = 8 per treatment group) were dosed daily for one year with either 25 or 50 mg/kg MXC (98% pure, ICN Biomedicals Inc., Aurora, OH), 0.5 mg/kg DES (ICN Biomedicals Inc., Aurora, OH), or vehicle control. Dosing was conducted 1300–1430 h, about 6 h after the morning feeding and 2 h before the afternoon feeding.

Doses were selected based on previous studies. The 0.5 mg/kg dose of DES had previously been shown to affect offspring reproductive tract when administered to pregnant rhesus (Hendrickx *et al*., 1979, 1987–88). The 25 and 50 mg/kg doses of MXC were the lowest effect estrogenic doses in rodent studies (Chapin *et al*., 1997; Gray *et al*., 1988b, 1989). Estrogenicity was confirmed in a pilot study, which examined changes in vaginal epithelial smears when the agents were administered to mature female monkeys during the luteal phase of the menstrual cycle.

Doses were individually weighed and mixed with fruit-flavored baby food (Gerber Products, Fremont, MI) in a syringe (3 cc or 6 cc) and administered orally. The dose was based on the animal's previous bimonthly body weight. Monkeys were trained to come to the front of the cage and sip from the tip of the syringe. If monkeys did not accept the baby food directly from the syringe, the dose was placed in a well on a plastic bar, which fit between the cage bars and attached to the front of the cage. If the dose was not consumed after 1 h, the bar was removed from the cage, and the amount remaining was recorded. If, after 3 days, monkeys still did not consume all the baby food from the plastic bar, the dose was placed in a marshmallow or piece of fruit. A dosing aversion developed among some of the animals as the dosing period progressed. All animals readily accepted the baby food until around the fourth month of dosing. By the end of dosing, six DES-treated, four MXC25-treated, and two MXC50-treated monkeys, and zero controls did not use the feeding syringe one or more times in a month, but were instead given their dose from the feeding bar or in fruit or marshmallow.

Vaginal bleeding. Monkeys were monitored visually for the presence of blood around the perineal area and under the tail by project investigators. During the dosing and recovery period they were checked three times per week, except during the 2 weeks following the end of dosing, when they were checked daily. If necessary, animals were confined to the front of the cage with a retractable rear cage wall and a flashlight was used to better visualize the area. However, many animals learned to present their rump and raise their tail for the visual menses checks. In addition to checks by investigators, observations were made daily by animal care technicians for the purpose of following menstrual cycles. Both types of data were used for menarche determination.

Serum estrogen receptor alpha (ERα) activation assay. A study was conducted at the end of the dosing period to assess the levels of ERα-mediated estrogenic activity present in the animals' serum. Successive animals were dosed at 5 min intervals in the morning to allow precise time-delays for the later blood sampling. The previous dose was given 23–24 h prior to the test dose. The time at which the dose was completely consumed was recorded as the base time. Three 3-ml blood draws were obtained at 2, 12, and 24 h after the base time without anesthesia by confining the monkey to the front of the cage and arm presentation outside the cage door. Samples were centrifuged, and the serum was removed and frozen at <−80°C until analyzed. Serum was analyzed for estrogenic activity using a transcription activation assay for estrogen receptor binding performed both before and after use of a polyclonal antibody to E2, which removes endogenous steroidal estrogen activity (Natarajan *et al*., 2002).

The bioassay detected estrogen-dependent activation of gene expression (Rogers and Denison, 2000). It used human ovarian carcinoma cells (BG1) that have been stably transfected with a luciferase reporter gene plasmid under the regulation of four estrogen-response elements. These cells are capable of detecting estrogenic chemicals by ERα-mediated transactivation of the reporter gene. The cells contain mRNA for both ERα and ERβ, but only the protein for ERα can be detected by Western blot (Rogers and Denison, 2000), indicating that the assay detects estrogenic activity mediated primarily by ERα.

The bioassay was performed as previously described with slight modification. Briefly, transfected BG1Luc4E2 cells were grown in normal cell culture medium (MEM with 10% FBS). When the cells reached approximately 90–95% confluency, they were washed with phosphate buffered saline, trypsinized for 30 seconds, dispersed with cell culture medium and plated in 150-mm

dishes. The next day, following plating, the media was removed and was overlaid with estrogen-depleted media (phenol-red free media, Sigma, St. Louis, MO), supplemented with 10% charcoal-Dextran treated fetal bovine serum (FBS) (Hyclone, Logan, UT)). The cells were maintained in the estrogen-depleted media for 6 days, dispersed with estrogen-depleted media, and then plated in 96-well microtitre plates. After 24-h incubation, media was removed and the cells were overlaid with 250 μl of estrogen-depleted media containing increasing concentration of each of E1 and E2 at 1% final volume and incubated for an additional 24 h. The media was then removed and the cells were lysed using 100 μl of 1X lysis buffer (Promega, Madison, WI). Luciferase activity in 45 μl lysate was measured in a Dynatech ML2550 microtiter-plate luminometer, following the addition of 100 μl of luciferase reagent with a delay time of 2 s and integration time of 2 s. Luciferase activity was expressed as relative light units (RLU)/mg protein. The minimal detection limits were 1 pM for E2, and 10 pM for E1. To detect the estrogenic activity of serum, cells were maintained as above, and after an initial 24-h incubation, media was removed, and the cells were overlaid with 250 μl of estrogen-depleted media containing estradiol standards and test serum at 1% final volume, incubated for an additional 24 h, and the luciferase activity was determined as above.

Observations of sex skin swelling/reddening. Skin in certain delimited areas (perineum, trunk, face) may swell and redden in response to estrogen in rhesus monkeys. The swelling is due to the presence of estrogen receptor (ER) in dermal fibroblasts (Bentley *et al*., 1986). The presence and extent of sex skin was recorded once a week beginning 6 weeks after onset of dosing, the time when sex skin was first seen in the young monkeys. Monkeys were observed in their home cages by investigators trained for reliability. Primary sex skin in the perineal area (groin and rump) was rated according to the severity of redness and swelling (either slight, moderate, or extreme). Nonperineal sex skin (face, sides, arms, legs, back, abdomen, and base of the tail) with redness or swelling was also noted and rated.

Growth and gender related morphology. Weights were obtained at bimonthly intervals throughout the study using standard procedure for the colony. Food restriction was not used prior to weighing. A transfer cage, which the monkeys had been taught to enter, was attached to the door of their home cage. The weight was then obtained to the nearest gram on a calibrated scale, and the weight of the transfer cage subtracted to obtain the monkey′s body weight. In the 2 months immediately following discontinuation of dosing, animals were weighed weekly.

Morphometric examinations were conducted under ketamine anesthesia (10 mg/kg i.m.) before treatment and 6, 12, and 20 months after initiation of treatment. Head, arm, and thigh circumferences were obtained with a plastic tape measure designed for use with human newborns. Skinfolds were measured at four locations (triceps, thigh (lateral aspect), suprailiac, and back (subscapular)) using Harpenden skinfold calipers (Country Technologies, Inc.). Three measures were obtained, and the median was used for each animal. Crown-rump was measured with the monkey's trunk in the natural flexed position. Long bone lengths (femur, tibia, humerus, radius, ulna) were measured from radiographs of the limbs obtained at this time. Nipple length was measured from the base to the tip of the left nipple; nipple width was measured as a diameter of the base using vernier calipers (Bel-Art Scienceware, Pequannock, NJ). Anogenital distance was measured from the end of the vaginal opening to the proximal end of the anus using calipers.

Ultrasound examination of the reproductive tract. Abdominal/pelvic ultrasound examinations were performed under ketamine anesthesia (10 mg/kg i.m.) at the end of the study to assess uterine size (length, width, and height), shape, contour, texture, presence of a uterine cavity, and endometrial thickness, as previously described (Tarantal, 1992). Size and shape of the ovaries were also recorded, and any atypical abdominal or pelvic structures were documented and measured.

Ovarian hormone cycles. Urine samples were collected for 60 consecutive days at the end of the study to examine ovarian cycles via estrogen and progesterone metabolites. Sample collection began on the animal's first day of menses in a designated month. Pans were placed under the cages in the afternoon, and urine was collected the next morning. Animals were not paired overnight during sample collection. The urine was centrifuged, and approximately 3 ml of the supernatant collected and placed in a cryogenic vial. Samples were frozen at <-80°C until assay using previously established methods (Shideler *et al*., 1993).

Statistical analysis. Data were analyzed with Statview (Version 5, Abacus Concepts, Berkeley, CA). For most measures, ANOVA was conducted with post hoc comparison of treated with control groups. The Mann-Whitney *U* test was used for nonnormally distributed data.

RESULTS

Estrogenicity of Exogenous Estrogen Dose Levels

The results from the *in vitro* ERα activation assay before and after precipitation of steroidal estrogen are shown in Figure 1. The total estrogenicity comparison indicated that serum from both MXC- and DES-treated animals had increased ability (about 50% higher) to activate gene expression through the ERa receptor compared to controls. The exogenous estrogen comparison indicates that serum from untreated controls had no estrogenic activity after precipitation of steroidal estrogen, while that of the treated groups was estrogenic. The exogenous estrogen activity in the treated monkeys declined slightly but significantly after the daily dosing of MXC/DES, although most of the activity remained at 24 h, suggesting the possibility of cumulative effects (Fig. 1, inset). It is not possible to derive an estimate of endogenous estrogenicity from these data, because interactions of receptor binding and activation prevent an assumption of additivity between endogenous and exogenous estrogens.

Growth

Body weights throughout the study are shown in Figure 2 (top panel). The DES group lost weight during the second 6-week period of treatment and failed to show weight gain comparable to control and MXC groups until the end of treatment, when weight gain resumed at a rate comparable to controls. However, the mean weight of the DES group remained significantly lower than that of controls for the duration of the study.

Food intake measures were also taken once or twice a week throughout the study (Fig. 2, bottom panel). Food intake was generally lower in the DES group during treatment, but did increase over time. The increase in food intake without a corresponding increase in weight led us to examine food use efficiency, the ratio of weight gained during the growth spurt to food intake during that period. There was a significant treatment effect on this ratio ($F = 9.568$, $p = 0.0002$) with the DES group ratio lower than that of controls ($p = 0.0002$), indicating lower food use efficiency.

Figure 3 shows weight gain during the growth spurt (study month 4–10) in 6-week periods, the shortest interval that provides reliable growth estimates for pubertal rhesus females (Blackwelder and Golub, 1996). The DES group gained sig-

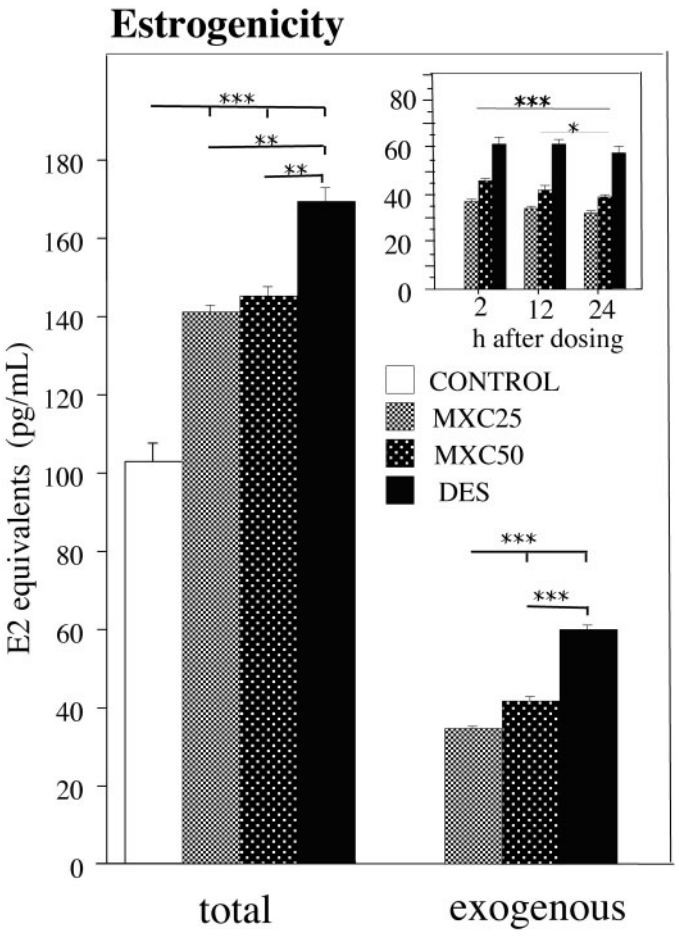

FIG. 1. ERα-mediated estrogenicity of serum as evaluated with a cell-based *in vitro* receptor activation assay. Each monkey is represented by the mean of three samples obtained 2, 12, and 24 h after dosing. Exogenous estrogen values were obtained after immunoprecipitation of steroidal estrogen from the serum samples. ANOVA were significant for total ($F = 22.93$, $p < 0.0001$) and exogenous ($F = 324.12$, $p < 0.0001$) estrogen activity. In post hoc tests for total estrogenicity, each group differed from controls ($p < 0.0001$), and in addition, the DES group differed from each of the MXC groups ($p < 0.01$). For exogenous estrogenicity, each of the treated groups (DES, MXC25, MXC50) differed significantly from one another ($p < 0.001$). The inset demonstrates the effect of time-after-dosing on exogenous estrogenicity ($F = 8.81$, $p = 0.0005$; 2 versus 24 h $p = 0.0001$; 12 versus 24 h $p = 0.02$). There was no interaction between group and time-after-dosing. Group mean ± SEM are shown. *$p < 0.05$, **$p < 0.01$,***$p < 0.001$ compared to control group.

nificantly less weight than controls at all time points until the discontinuation of treatment. The MXC25 group lagged behind controls in weight gain at the peak of the growth spurt ($p = 0.05$) and the MXC50 group toward the end of the growth spurt ($p = 0.02$). Although the DES group gained little weight, they did demonstrate a very attenuated "growth spurt" at the same time as the control monkeys.

Data on linear growth (trunk length or crown-rump length, long bone length) were obtained at the beginning and end of treatment and at the end of the recovery period (Fig. 4). A ″height″ measure was obtained as the sum of crown-rump, femur, and tibia lengths. Height growth was clearly depressed in the DES group during treatment, and DES monkeys remained shorter than controls at the end of the study. Figure 4 shows that trunk length, as well as long bone length, was retarded in growth. In addition, the slope of height increase during dosing appeared lower in the MXC groups. Additional analysis demonstrated less height increase (end of treatment − baseline) in the MXC25 ($p = 0.05$) as well as the DES ($p < 0.001$) group compared to controls. Trunk length appeared more affected by MXC than long bone growth.

DES-treated monkeys were also significantly smaller than controls and MXC groups in arm and thigh circumference (muscle mass) and skin fold thickness (body fat) at the end of

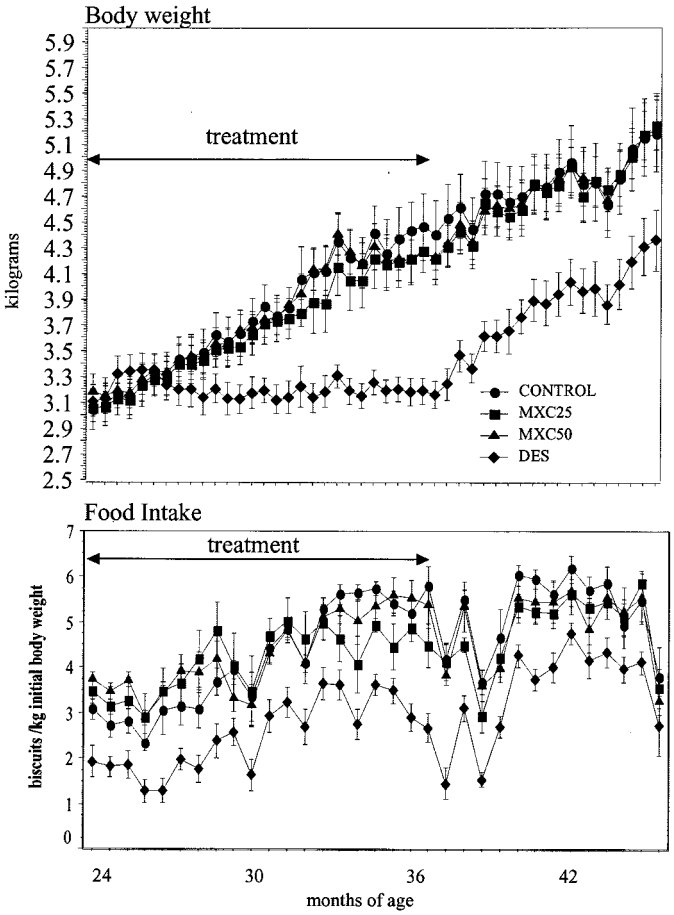

FIG. 2. Changes in body weight and food intake during and after exogenous estrogen treatment in pubertal female rhesus monkeys. Bimonthly weights and food intake measures are shown. There were treatment effects on body weight during ($F = 3.449$, $p = 0.03$, control versus DES $p = 0.009$) and after ($F = 5.284$, $p = 0.0052$, control versus DES, $p = 0.0021$) dosing. In addition, body weight increased during these periods ($p < 0.001$), with a significant group × time interaction ($p < 0.001$), reflecting the reduced weight gain in the DES group during dosing and the greater weight gain after discontinuation of dosing. Food intake (biscuits food/kg body weight) ANOVA result: during dosing $F = 4.8$, $p = 0.012$, control versus DES $p < 0.002$, control versus MXC25 and MXC50, $p < 0.01$; after dosing $F = 3.6$, $p = 0.030$, control versus DES $p < 0.01$, control versus MXC50 $p < 0.05$.

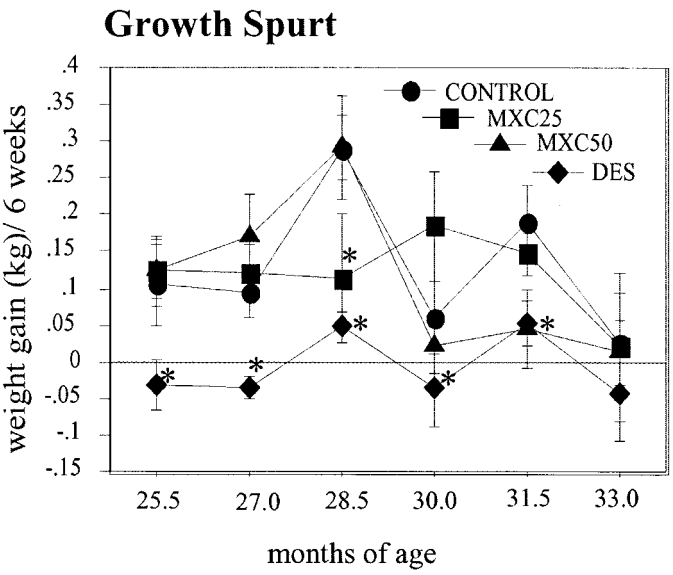

FIG. 3. Comparison of timing and extent of the pubertal growth spurt in rhesus monkeys treated with exogenous estrogen. Weight gain over six week periods from 3 months before to 3 months after the expected age at menarche is shown. *Different from control, $p < 0.05$.

dosing (data not shown). This suggests a proportional decrease in growth across body compartments. Muscle mass and body fat measures were no different from controls after recovery, although weight and height (crown rump and femur lengths) were still significantly smaller in the DES group than in controls.

Secondary Sex Characteristics

Increase in nipple volume (calculated from width at base and the length) is the major external secondary sex characteristic to develop in rhesus monkeys at puberty. Nipple size increased eightfold in controls from the beginning to end of the treatment period. The nipple volume was significantly smaller in the DES group 9 and 12 months after initiation of treatment and after recovery, and marginally significantly smaller ($p = 0.048$) in the MXC50 group at the end of treatment (Fig. 5). Eight months after treatment, nipple size had further increased but was still significantly lower than controls in the DES group (Fig. 5).

Another puberty-related morphological change is a decrease in anogenital distance due to an increase in the length of the vaginal opening. Anogenital distance decreased about 3 cm (22%) in controls from the beginning to the end of treatment ($F = 2.47$, $p = 0.043$, paired t-test) but did not change significantly in the other groups (data not shown). At follow-up 8 months after treatment was discontinued, there were no group differences in anogenital distance or change in anogenital distance from baseline.

A third secondary sex characteristic, sex skin swelling and reddening, appears after puberty. Female monkeys demonstrate sex skin in the perineal area and, less frequently, on the face and sides (nonperineal sex skin). Figure 6a indicates that nonperineal sex skin was more frequently detected in the DES group, as well as the MXC25 group, than in controls. The MXC50 group was less affected. Sex skin on the sides of the trunk was detected in monkeys treated with exogenous estrogens, but not in controls (Fig. 6b).

In contrast, occurrence of perineal sex skin swelling was not significantly affected during treatment. Sequential change in sex skin swelling in the rump area during treatment was examined to determine possible premature appearance in exogenous estrogen groups (Fig. 6c). Occurrence of swelling increased in controls throughout the dosing period. The DES group had a greater incidence of swelling early in the dosing period. A similar trend was seen in the MXC25 group. However, as swelling plateaued in controls, the incidence in the DES group declined and remained lower than normal after dosing was discontinued.

Menses/Menarche

Menarche was defined as the first day in the treatment period when vaginal bleeding was confirmed by observation. All control monkeys attained menarche during the treatment period. Four of the eight monkeys in the DES group did not show any vaginal bleeding during treatment, and three others showed only one day (Table 1). The average number of days with menses during treatment was significantly lower in the DES monkeys than controls ($F = 7.19$, $p = 0.001$, control versus DES $p = 0.0002$). The number of days from the beginning of treatment to the first instance of vaginal bleeding is also shown in Table 1. Values were somewhat higher in the MXC50 group and considerably higher in the DES group than in controls. The comparison between control and DES groups was significant with nonparametric testing (Mann-Whitney $U = 3$, $p = 0.0023$). The number of months of age at first vaginal bleeding showed a similar pattern to number of days dosing at menarche.

Vaginal bleeding as an index of menarche is complicated by the suppression of uterine bleeding by exogenous estrogen. In order to examine whether lack of vaginal bleeding indicated failure of ovarian cyclicity to mature, or suppression of menses, we looked at vaginal bleeding immediately after discontinuation of DES. The four DES monkeys who had not yet reached menarche demonstrated vaginal bleeding 2, 3, 5, and 5 days after discontinuation of treatment, and the four DES monkeys who had previously demonstrated some menses had vaginal bleeding 3, 4, 7, and 11 days after discontinuation of treatment. This suggests that vaginal bleeding was suppressed; however, it is not possible to determine whether menarche was also delayed.

Figure 7 is a graphical presentation of the relative frequency of months with vaginal bleeding in the various groups in terms of the cumulative group totals. Vaginal bleeding was sup-

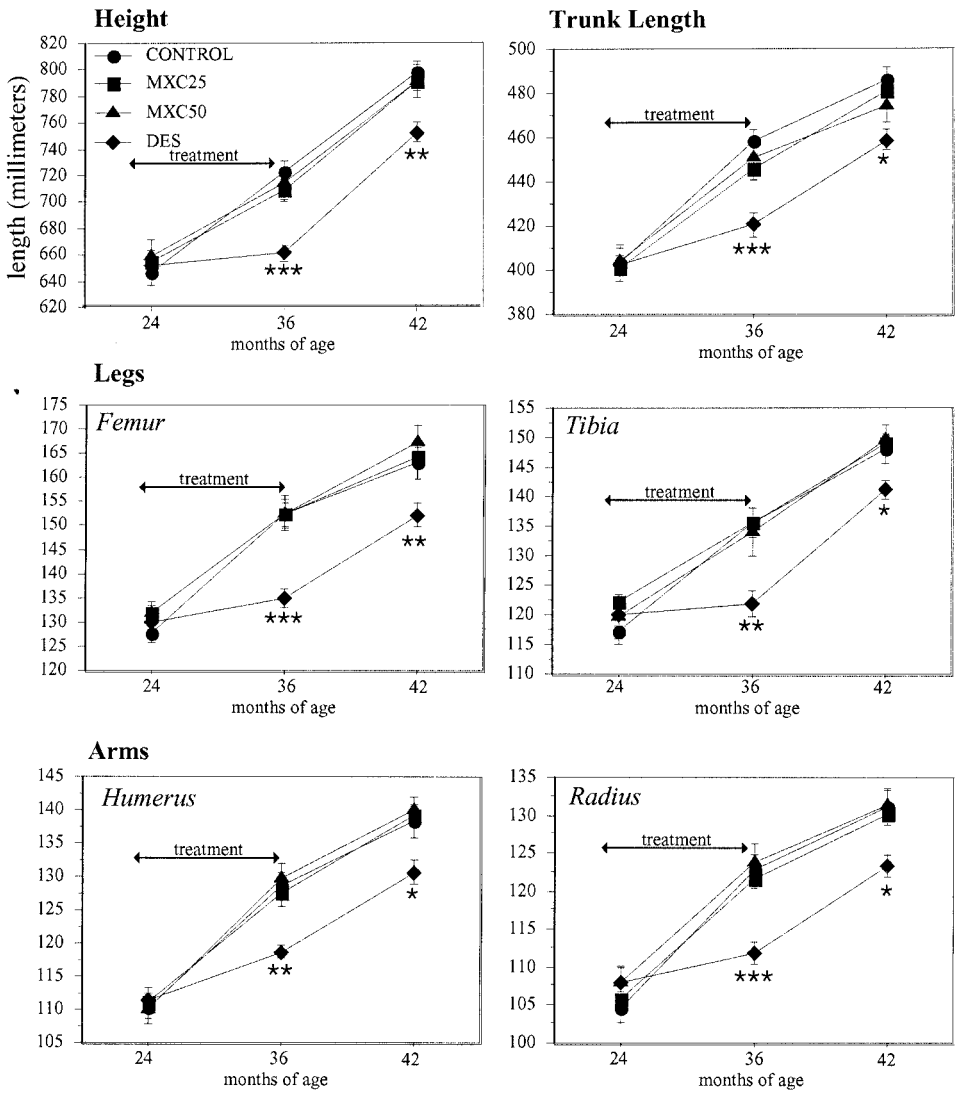

FIG. 4. Linear growth in pubertal rhesus monkeys treated with exogenous estrogen. Height = trunk length (crown rump) + humerus + tibia. ANOVA results: group × period, $p < 0.001$ for all measures; post hoc comparison with control. *$p < 0.05$, **$p < 0.01$,***$p < 0.001$.

pressed in the DES group during treatment but was similar to controls thereafter. The MXC groups did not clearly show the decrease in cycling typically seen during the summer months in rhesus monkeys (months 15–18 of the study).

Reproductive Tract

In ultrasound examinations after the recovery period, enlarged ovaries and ovarian cysts or solid tissue masses were noted in a number of cases: 2/8 controls, 5/8 MXC25, 2/8 MXC50, and 5/8 DES-treated animals. Uterine size and endometrial thickness as determined by ultrasound were not significantly different across groups (Table 2). Immature prepubertal appearance of the uterus was noted in two monkeys, one in the control and one in the MXC50 group. Uterine size was not correlated with body weight ($r = -0.30$), but appeared to be related to ovarian size and ovulatory status. Monkeys demonstrating anovulatory cycles (see section below on ovarian hormone cycles) had the smallest uteri (area computed from length, width, and height < 5 cm^3), while monkeys with two enlarged ovaries, one very large ovary, or ovarian cysts/masses had the largest uteri (>9 cm^3).

Ovarian Hormone Cycles

Ovulatory cycling was examined at the end of the recovery period through analysis of daily urine samples for 60 consecutive days, using antibody for estrogen and progesterone urinary metabolites. Presence of ovulatory cycles and the duration of the luteal and follicular phases were categorized by an experienced investigator (BLL) (Table 3). Despite the small numbers of animals, some trends were indicated. There were fewer monkeys with normal cycles in the treated groups than in the control group. MXC-treated monkeys tended to exhibit short follicular phases (estrogen peak < 11 days post menses), while the DES group had short luteal and long follicular stages.

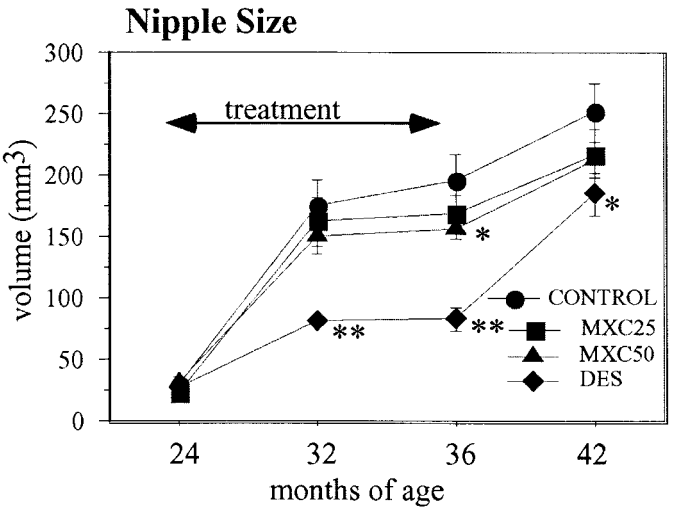

FIG. 5. Changes in nipple volume in pubertal rhesus monkeys treated with exogenous estrogen. ANOVA result: before treatment $F = 0.7$, ns; at the end of treatment $F = 12.3$, $p < 0.001$, control versus DES, $p < 0.001$, control versus MXC50, $p < 0.05$; after a 6-month recovery period, $F = 2.1$, $p < 0.12$, control versus DES, $p < 0.02$. $*p < 0.05$, $**p < 0.01$,$***p < 0.001$ compared to control group.

A limited number of monkeys showed anovulatory cycles, and this did not differ by group. One monkey in the control group showed no ovulatory cycles and no menses during the urine sampling period or the subsequent twelve months. This monkey mensed on only 1 day during the study, and also had a small uterus. Colony records indicate that she did mense regularly during the next breeding season.

DISCUSSION

Most markers of puberty in the study were affected by DES during the treatment period, with residual effects seen after recovery. MXC treatment did not affect growth or vaginal bleeding during treatment but did influence sex skin swelling. Marginal effects of MXC were seen on height and nipple growth and timing of the growth spurt. After discontinuation of treatment and 6–8 months of recovery, effects of both agents on ovarian structure and cyclicity and on incidence and location of sex skin swelling were recorded.

The issue of precocious and delayed puberty is prominent in the endocrine disruption literature but is difficult to address when estrogenic agents are administered during puberty. In the absence of exogenous estrogen, a series of biological markers (pubertal growth, menarche, secondary sex characteristics) are synchronized by the onset of ovarian estrogen production. However, if exogenous estrogen is present during this period, markers of sexual development can be dissociated from their normal sequence by the direct action of the exogenous estrogen on peripheral tissues. Thus, when exogenous estrogen treatments are used in puberty, it is not possible to infer the timing of sexual maturation, as controlled centrally by hypothalamus via ovarian estrogen production, from external markers of puberty like menses and breast development in humans and anogenital distance and vaginal opening in rodents.

Growth restriction in the DES group was a striking finding in our study. Possible mechanisms include reduced food intake and/or decreased metabolism, a common estrogenic action

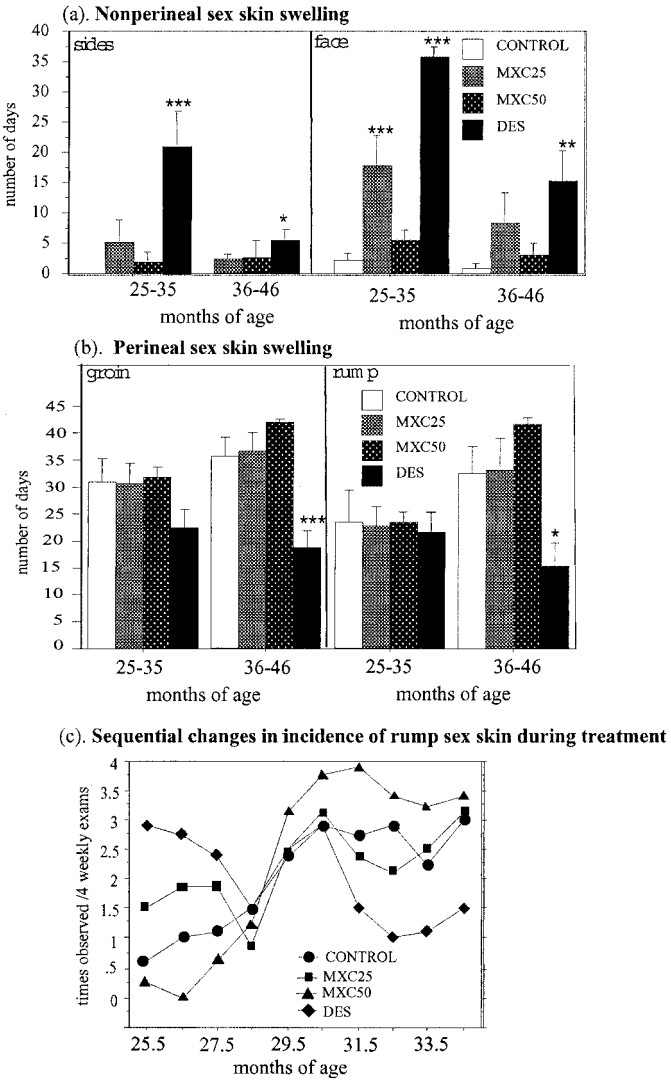

FIG. 6. Incidence of sex skin swelling and reddening before and after dosing of pubertal rhesus monkeys with exogenous estrogen. Group mean ± SEM are shown. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ compared to control group. (a) Nonperineal sex skin swelling and reddening. ANOVA results: During dosing: Face $F = 27.7$, $p < 0.0001$, control versus DES $p < 0.0001$, control versus MXC25 $p = 0.0007$; Sides $F = 8.65$, $p = 0.0003$; control versus DES $p = 0.0003$. After dosing: Face $F = 3.0$, $p = 0.047$, control versus DES $p = 0.010$; Sides $F = 1.84$, ns. (b) Perineal sex skin swelling. ANOVA results: During dosing: Groin $F = 1.5$, ns; Rump $F = 0.1$, ns. After dosing: Groin $F = 11.9$, $p < 0.001$, control, MXC25 and MXC50 versus DES $p < 0.001$; Rump $F = 5.7$, $p = 0.003$, control, MXC25 and MXC50 versus DES $p < 0.02$. (c) sequential changes in incidence of sex skin swelling in the rump area. Each block represents a 4-week period. Group means are shown.

TABLE 1
Onset of Menses during Treatment of Pubertal Rhesus Monkeys with Exogenous Estrogens

	Control $N = 8$	MXC25 $N = 8$	MXC50 $N = 8$	DES $N = 8$
Age at first menses (months)	28.4 ± 0.7[a]	30.4 ± 1.3	30.3 ± 0.6	34.5 ± 1.3*
Weight at first menses (kg)	3.5 ± 0.2	3.4 ± 0.1	3.6 ± 0.1	3.2 ± 0.1
Time to first menses from treatment onset (months)	3.4 ± 0.7	3.6 ± 0.5	5.2 ± 0.6	9.2 ± 1.3***
Days with menses during treatment	16 ± 10	21 ± 8	16 ± 6	0.9 ± 1.3*
Time to first menses from treatment offset (days)	17 ± 40	22 ± 3	17 ± 2	5 ± 1**

[a]mean ± SEM.
*Significantly different from controls, post-hoc *t*-test $p = 0.0002$.
**Significantly different from controls, post-hoc *t*-test $p = 0.004$.
***Significantly different from controls, Mann-Whitney U, $p = 0.0023$.

(Wade and Gray, 1979; Wade *et al*., 1985), earlier age at epiphyseal closure (Cutler, 1997), and feedback suppression of GnRH, which plays a role in the pubertal growth spurt (Mauras *et al*., 1996). Both food intake and food use efficiency were influenced by DES in our study, but height growth was concomitantly reduced. Further, the pattern of growth retardation in this study differed from that in a previous study (Golub *et al*., 1997), in which leuprolide, a GnRH suppressing drug, was administered to monkeys from 18 to 30 months of age. In that study, food intake was not suppressed, linear growth was less affected than weight gain, and muscle mass was the most influenced body compartment. Thus, information from the present experiment does not clearly identify the mechanism of DES-induced growth restriction. As regards MXC, some effects on growth were indicated in the present study in terms of delays of body weight gain during the growth spurt and trunk growth during the treatment period and altered timing of the peak of the growth spurt. MXC-induced growth retardation has been reported in rats (Chapin *et al*., 1997; Gray *et al*., 1999).

Long-term effects of exogenous estrogen on ovarian structure were suggested. Without histological examination, it is not possible to understand the nature or consistency of the underlying changes in ovarian cells. MXC dosing (32 mg/kg) in young adult (39-day-old) mice has been shown to increase ovarian epithelial height as well as to promote antral follicle atresia (Borgeest *et al*., 2002). DES in drinking water (6.5 mg DES/kg/day), in rats from 21 to 100 days of age, led to smaller ovaries; no histological examination was done (Odum *et al*., 2002).

There were indications of abnormalities in ovarian cyclicity in this study, with shorter follicular phases (<11 days) seen in MXC groups. Follicular phase of ovarian cycles in rhesus monkeys are similar to those of humans and average 14 days in length (Potter *et al*., 1999). In women, 7% of cycles have short follicular phase (<11 days) (Adams *et al*., 2001). There are several lines of evidence in humans of an association between a short follicular phase, as seen in MXC, and infertility. Older women have an earlier rise in FSH, a shorter follicular phase, and reduced fertility (Klein *et al.,* 2002). Women with short follicular phases (estradiol peak < 11 days after menses) had a 50% reduction in estimated 12-month pregnancy rate com-

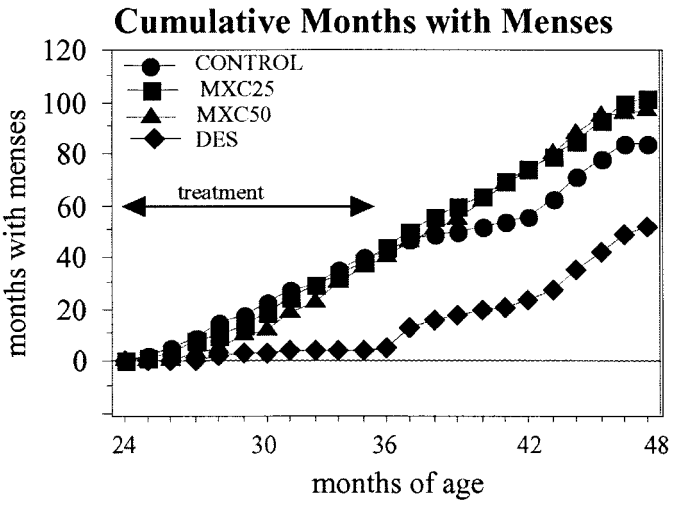

FIG. 7. Cumulative number of months with vaginal bleeding occurring at least once during the month. (Data from daily menses checks were used to construct this graph. The presence of menstrual cycles or ovulatory cycles is not confirmed with this method.)

TABLE 2
Uterine Measures Obtained by Ultrasound in Rhesus Monkeys Exposed to Exogenous Estrogen during Puberty and Examined after an 8-Month Recovery Period

	Control	MXC25	MXC50	DES
Uterine length (mm)	43.5 ± 1.0*	45.9 ± 1.9	43.7 ± 1.6	41.9 ± 1.9
Uterine volume (cm^2)	5.8 ± 0.7	7.4 ± 1.2	5.1 ± 0.9	5.3 ± 0.8
Endometrial thickness (mm)	2.9 ± 0.2	2.9 ± 0.2	2.2 ± 0.3	2.7 ± 0.2

There were no statistically significant differences between control and treated groups.
*Mean ± SEM, n = 8 per group

TABLE 3
Ovarian Hormone Cycles Characterized from Urinary Metabolites 6–8 months after Cessation of Treatment

				Type of abnormal phase lengths			
				Follicular		Luteal	
	Normal	Abnormal phase length	Anovulatory	Short	Long	Short	Long
control	5*	2	1	0**	1	1	0
MXC25	3	3	2	3	0	0	1
MXC50	0	8	0	4	3	2	0
DES	1	6	1	1	3	3	0

*Number of monkeys, n = 8 per group.

**Number of monkeys with at least one cycle demonstrating this type of abnormal phase length.

pared to controls (Check *et al*., 1992). Other studies set the length of the follicular stage to either 7 or 14 days using hormones in infertility patients and found that uterodome formation in the uterus, a marker of implantation readiness, was lower with the 7 day follicular phase (Adams *et al*., 2001). Luteal phase deficiency, as seen in the DES group, is also a concern for fertility.

Sex skin swelling may be a useful marker of estrogenic actions in macaque monkeys. Estrogen receptors in perineal sex skin have been localized to fibroblasts, which regulate swelling by induction of hyaluronic acid synthetase (Bentley *et al*., 1986; Onouchi and Kato, 1983; Uzuka *et al*., 1981; West *et al*., 1990). Progesterone leads to detumescence during cyclical sex skin swelling. Sex skin is not a readily available indicator of estrogen response in humans and laboratory rodents, although estrogen receptors are also found in the skin of these species, and sex skin swelling is present at estrus in rats (Coppola and O'Connell, 1989; Haczynski *et al*., 2002; Hasselquist *et al.*, 1980).

The results of the present study contribute to a small literature on MXC and DES administration during puberty to female rats. A large systematic study with DES administration in drinking water (1.5–6.5 μg/kg/d) (Odum *et al*., 2002) found growth retardation, advanced age at vaginal opening, and fewer full estrus cycles in young adults. These effects could be compared to growth retardation, early appearance of sex skin swelling, and reduction of menses in the present study. The highest dose DES group in the rat study demonstrated a reduction of ovulation (corpora lutea) and also had uterine dilatation and squamous metaplasia. In a MXC study Gray *et al.* (1989) administered 25 and 50 mg/kg MXC to female Long-Evans rats by gavage from 21 days of age through puberty, mating, and pregnancy. Vaginal opening and appearance of the first estrous vaginal smear were accelerated at 25 and 50 mg/kg/d. The first full estrus cycle was also accelerated in the MXC 25 mg/kg/d group. All females were fertile and delivered their first litter at similar ages. No histologic effects on ovaries were noted when female rats were killed at 80–85 days of age.

A potentially important finding of the present study was the apparently greater estrogenic effect of the lower dose of MXC on some endpoints (height growth, early appearance of perineal sex skin, incidence of nonperineal sex skin during treatment, and ovarian structural changes). The lower MXC dose (25 mg/kg/d) was more similar to DES than the higher MXC dose (50 mg/kg/d). One possibility for similarity between low dose MXC and DES is the relative doses at which MXC, via HPTE, exerts agonist versus antagonist activity at ERα and ERβ receptors. Although both DES and MXC (via its metabolite HPTE) bind to and activate estrogen receptors, they differ from E2 and from each other in the profile of their biological actions relevant to endocrine effects. Prior research has explored central (brain) versus peripheral effects (Gray *et al*., 1988, 1999), and differential steroid receptor (estrogen, androgen, progesterone) binding and activation. Another consideration is differential ERα and ERβ binding and activation (Giguere *et al*., 1998; Mauras *et al*., 1996; Tremblay *et al*., 2001). These two receptors, products of different genes, differ in their tissue distribution (Byers *et al*., 1997; Haczynski *et al*., 2002; Osterlund *et al*., 1998; Pelletier and El-Alfy, 2000; Shughrue *et al*., 1998; Shugrue *et al*., 1996) and expression during pubertal development (Wilson *et al*., 1998). Although both receptors act at the same gene promoter regions, they often have opposite actions, suggesting the importance of a balance between their activation in mediating biological function. ERα and ERβ mRNA tissue distribution has been studied in monkey as well as in rodents and humans (Goldsmith *et al*., 1997; Pau *et al*., 1998; Pelletier *et al*., 1999).

DES and MXC differ from E2 in their relative receptor binding at ERα and ERβ; while DES binds ERα with about fivefold higher affinity than E2, binding at ERβ is only about twofold higher. MXC also has a lower relative binding affinity than E2 at ERβ versus ERα and has the interesting property of acting as an agonist at the ERα and an antagonist at ERβ (Gaido *et al*., 1999, 2000; Waters *et al*., 2001). Exogenous estrogens have also been shown to differentially bind two identified ERβ isoforms (Petersen *et al*., 1998) and to interact with estrogen-related receptors (ERRs) (Tremblay *et al*., 2001).

In the present study, there was considerable intragroup variability in response to exogenous estrogen treatment. Although rhesus monkeys are a standard laboratory model for human health research, they are not selectively bred and demonstrate genetic variability. It is possible that some monkeys, like humans, have genetic polymorphisms that influence pubertal development or xenobiotic metabolism. In particular polymorphisms of the ERα receptor have been shown to contribute to age at menarche in girls (Stavrou *et al*., 2002). Understanding of genetic predisposition to toxicant action would be valuable in generalizing from animal to human situations and could be pursued in nonhuman primate models.

Given that treatment-related effects were detected 8 months after cessation of dosing, it will be important to determine if reproductive function is affected in this cohort of monkeys. This information will be relevant to human populations exposed to estrogenic drugs or pollutants during puberty.

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